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Full-length Prepro-α-Factor Can Be Translocated across the Mammalian Microsomal Membrane only if Translation Has Not Terminated

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Abstract. We have previously shown that fully synthesized prepro-α-factor (ppαF), the precursor for the yeast pheromone α-factor, can be translocated posttranslationally across yeast rough microsomal (RM) membranes from a soluble, ribosome-free pool. We show here that this is not the case for translocation of ppαF across mammalian RM. Rather we found that a small amount of translocation of full-length ppαF is observed, but is solely due to polypeptide chains that were still ribosome bound and covalently attached to tRNA, i.e., not terminated. In addition, both signal recognition particle (SRP) and SRP receptor are required, i.e., the same targeting machinery that is normally responsible for the coupling between protein synthesis and translocation. Thus, the molecular requirements for targeting are distinct from posttranslational translocation across yeast RM. As termination is generally regarded as part of translation, the translocation of full-length ppαF across mammalian RM does not occur "posttranslationally," albeit independent of elongation. Most other proteins for which posttranslational translocation across mammalian RM was previously claimed fall into the same category in that ribosome attachment as peptidyl-tRNA is required. To clearly separate these two distinct processes, we suggest that the term posttranslational be reserved for those processes that occur in the complete absence of the translational machinery. We propose the term "ribosome-coupled translocation" for the events described here.

In higher eukaryotes, secretory and some integral membrane proteins are synthesized on ribosomes attached to the endoplasmic reticulum (ER)[1] membrane (Palade, 1975). This observation, together with the finding that efficient translocation could be obtained in vitro only for nascent proteins during their synthesis (Blobel and Dobberstein, 1975), led to the conclusion that translocation across mammalian rough microsomal membranes (RM) is a strictly "co-translational" process. The signal recognition particle (SRP) has a high affinity for ribosomes synthesizing secretory proteins (Walter et al., 1981) and in conjunction with the SRP receptor (Walter and Blobel, 1981; Gilmore et al., 1982*a*, *b*; Meyer et al., 1982) was determined to function as the adapter between the translation and translocation machinery, thus providing further support for this hypothesis.

In contrast, it has recently been reported that translocation can occur, albeit at reduced efficiency, for several fully synthesized proteins across mammalian RM after further translation has been inhibited with cycloheximide (Hansen et al., 1986; Caulfield et al., 1986; Mueckler and Lodish, 1986*a*, *b*; Perara et al., 1986; Chao et al., 1987). These findings led to the conclusion that translocation of proteins across mammalian ER membranes is not necessarily coupled to translation and, thus, would resemble the process of translocation across yeast ER membranes which can occur efficiently posttranslationally (Hansen et al., 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986). Paradoxically, in control experiments it was noted that SRP, thought to function to target the ribosomes synthesizing secretory proteins to the ER, appeared to be required for this elongation-independent translocation process across mammalian membranes (Hansen et al., 1986; Mueckler and Lodish, 1986*a*). In contrast, SRP, SRP receptor, or ribosomes do not appear to be required for posttranslational translocation across yeast RM. This prompted us to further analyze in molecular detail the translocation of full-length proteins across mammalian RM to address these apparent differences. To compare directly the requirements for translocation across the yeast and mammalian RM, we used prepro-α-factor (ppαF) as substrate since this preprotein retains its translocation competency after its termination when assayed for posttranslational translocation across yeast RM.

1. *Abbreviations used in this paper*: ER, endoplasmic reticulum; CTABr, hexadecyltrimethyl ammonium bromide; K-RM, salt-extracted rough microsomal membrane; pαF.3, glycosylated pro-α-factor; ppαF, prepro-α-factor; PRS, postribosomal supernatant; RP, ribosomal pellet; SRαf, signal recognition particle receptor α subunit; SRP, signal recognition particle; T-RM, inactive salt extracted rough ribosomal membrane after mild proteolysis with trypsin.

Materials and Methods

RM (Walter and Blobel, 1983*a*), salt-extracted RM (K-RM; Walter and Blobel, 1983*b*), trypsin-treated RM (T-RM; Gilmore et al., 1982*a*), SRP (Walter and Blobel, 1983*b*), and the 52-kD cytoplasmic fragment of the SRP receptor α subunit (SRαf; Siegel and Walter, 1985) were prepared as previously described. Synthetic ppαF mRNA (Hansen et al., 1986) was translated in a wheat germ extract (Erickson and Blobel, 1983). After 30 min, cycloheximide was added to 1 mM to inhibit further elongation. Translocation reactions were initiated by the addition of 5 equivalents (Walter and Blobel, 1980) of microsomal membranes (either RM, K-RM, or T-RM) and SRP and/or SRαf, and the incubation was continued for 30 min at 26°C. The total volume of each reaction was 50 μl, containing 40 μl of translation extract. The ionic conditions of the translocation reactions were kept constant in all cases. After the second incubation, the microsomal vesicles were collected by centrifugation through a 50 μl 0.5 M sucrose cushion as previously described (Hansen et al., 1986). The pellets (containing the RM fraction) were dissolved directly in sample buffer and subjected to SDS-PAGE on 10–15% gradient gels. The gels were exposed to X-Omat AR Kodak film after fluorography with 2,5-diphenyloxazole.

Precipitations of peptidyl-tRNA with the cationic detergent hexadecyltrimethylammonium bromide (CTABr) were carried out as previously described (Gilmore and Blobel, 1985). Deacylation of nascent chains was carried out by the addition of 0.1 N KOH and incubation at 37°C for 15 min; the solution was then neutralized with acetic acid before CTABr precipitation. The postribosomal supernatant (PRS) and ribosomal pellet fractions were prepared as described (Hansen et al., 1986).

Removal of the low molecular mass molecules for the energy requirement experiments was performed as follows. After synthesis of ppαF was inhibited by the addition of cycloheximide, a 0.5 ml translation reaction was chromatographed on a 5 ml Sephadex G25 column equilibrated in translation buffer without ATP, GTP, and creatine phosphate. New cycloheximide was added to the eluate, which was then aliquoted for translocation reactions.

Results

To determine the SRP dependence of the translocation of full-length ppαF, we chose the wheat germ translation system which lacks endogenous SRP (Walter and Blobel, 1980; Meyer et al., 1982). After translation of ppαF mRNA for 30 min at 26°C, further protein elongation was inhibited by the addition of 1 mM cycloheximide. After a second 30-min incubation in the presence of canine pancreatic RM (containing endogenous SRP), the membranes were sedimented by centrifugation and the pellet fraction subjected to SDS-PAGE. In the presence of RM (Fig. 1 *A*, lane *2*) we observed that a small fraction (~1–2%) of ppαF sedimented as the glycosylated form (termed pαF.3; note that in addition to glycosylation the signal sequence of translocated ppαF is cleaved by signal peptidase (Waters et al., 1988), indicating that it had been translocated across the lipid bilayer. Translocation was confirmed by the resistance of pαF.3, but not cosedimenting ppαF, to externally added proteases (Fig. 1 *A*, lane *3*). As expected, pαF.3 was completely digested by protease if the permeability barrier of the membrane was disrupted by the addition of detergent (Fig. 1 *A*, lane *4*). The identity of pαF.3 was further verified by demonstrating its sensitivity to endoglycosidase H (Fig. 1 *A*, lane *5*). The presence of ppαF in the pellet fractions (~5% of the total ppαF synthesized is sedimented) was likely due to nonspecific aggregation: it was observed even in the absence of added RM (Fig. 1 *A*, lane *1*), and the sedimented ppαF was completely susceptible to proteolytic degradation (Fig. 1 *A*, lane

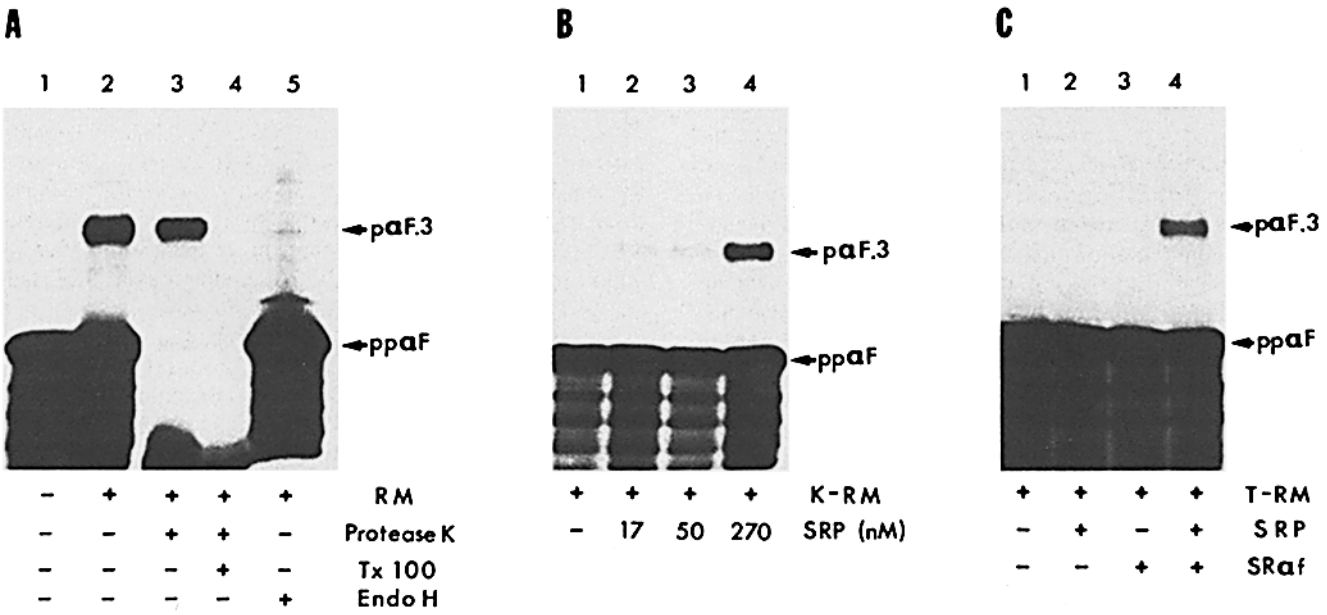

Figure 1. (*A*) Translocation of full-length ppαF across mammalian ER membranes can occur in the absence of elongation. Translocation reactions (see Materials and Methods) were carried out in the absence (lane *1*) or presence of 5 eq of RM (lanes *2–5*). Microsomal vesicles were then collected by centrifugation (see Materials and Methods) and subjected to SDS-PAGE. The samples in lanes *3* and *4* were treated with protease K before RM sedimentation (Hansen et al., 1986). Triton X-100 (*Tx 100*, 0.4%) was added together with the protease to the sample in lane *4*. The sample in lane *5* was treated with endoglycosidase H (*Endo H*; Hansen et al., 1986) after RM sedimentation. (*B*) Translocation of full-length ppαF in the absence of elongation is dependent on SRP. The reactions were carried out as in *A* with the exception that K-RM (depleted of SRP) were added instead of RM (lanes *1–4*). Purified SRP at 17 (lane *2*), 50 (lane *3*) or 270 nM (lane *4*) was added together with K-RM. (*C*) Translocation of full-length ppαF in the absence of elongation is dependent on SRP receptor. The reactions were carried out as in *A* with the exception that T-RM (depleted of SRP and SRαf) were added (lanes *1–4*). SRP was added (225 nM) to the reactions in lanes *2* and *4*. Purified SRαf (100 nM) was added to reactions in lanes *3* and *4*.

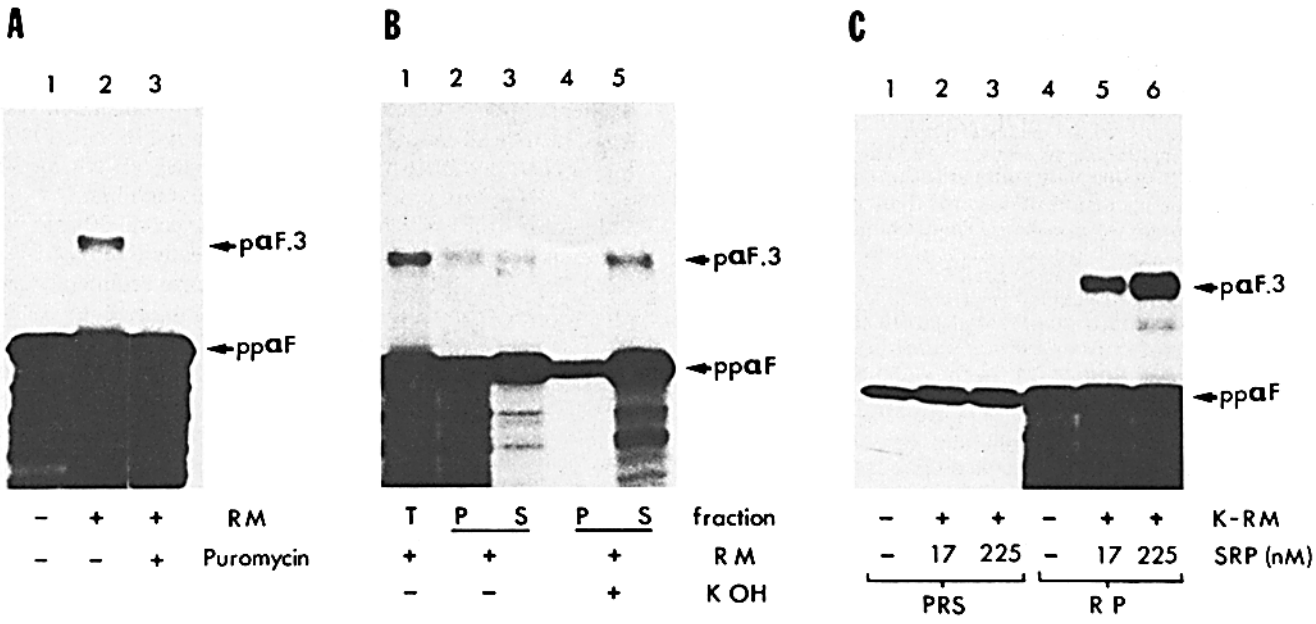

Figure 2. (*A*) Translocation of full-length ppαF cannot occur if translation is inhibited with puromycin. Translocation reactions were carried out as in Fig. 1 in the absence (lane *1*) or presence of RM (lanes *2* and *3*). Puromycin (1 mM) was added instead of cycloheximide to the reaction in lane *3*. To this reaction, RM was added after an additional 10-min incubation at 26°C in the presence of puromycin. (*B*) CTABr precipitation of the products of translocation in the absence of elongation. Reactions carried out as in Fig. 1 *A* were fractionated by CTABr precipitation. The total (*T*) products of reactions in the presence (lane *1*) of RM are shown. The CTABr pellets (*P*, lanes *2* and *4*) and supernatants (*S*, lanes *3* and *5*) of reactions identical to the one in lane *1* are shown. The samples shown in lanes *4* and *5* were deacylated by treatment with base before CTABr precipitation. (*C*) Translocation of ppαF in the absence of elongation is associated with the ribosomal fraction. After translation of ppαF the sample was fractionated into postribosomal supernatant (*PRS*) and ribosomal pellet (*RP*) fractions. The RP was resuspended in the same buffer and cycloheximide was added to both fractions. Translocation reactions were carried out as in Fig. 1 *A*. The reactions were adjusted such that the same amount of ppαF was present in each assay. K-RM at (5 eq/50 μl) was included in the reactions in lanes *2*, *3*, *5*, and *6*. SRP at 17 (lanes *2* and *5*)and at 225 nM (lanes *3* and *6*) was included. Control experiments in which no K-RM were added to PRS or RP are shown in lanes *1* and *4*, respectively. Note that only small amounts of ppαF are sedimented in the absence of RM from the PRS, since aggregated ppαF was largely recovered in the RP fraction.

3). Thus, the results presented in Fig. 1 *A* demonstrate that translocation of full-length ppαF across mammalian ER can occur (though only at 1–2% efficiency) in the absence of elongation as we previously noted (Hansen et al., 1986), although others failed to detect any translocation (Rothblatt and Meyer, 1986). It is important to note that if yeast RM instead of mammalian RM are added during the second incubation, ppαF is efficiently (30–50%) translocated as was previously described (Hansen et al., 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986). Hence, no factors that may be required for the posttranslational process were limiting in the extract.

We tested if this elongation independent translocation of ppαF across mammalian RM is dependent on the known components of the translocation machinery, such as SRP and SRP receptor. The results shown in Fig. 1 *B* indicate that the process is SRP dependent. When salt-extracted RM (K-RM), which are thus depleted of SRP, were added in the second incubation, no pαF.3 was obtained (Fig. 1 *B*, lane *1*). If, in addition to K-RM, the reactions were supplemented with increasing concentrations of purified SRP (Fig. 1 *B*, lane *3–4*) translocation was restored and correspondingly increasing amounts of pαF.3 were obtained. As would be expected from this result, we demonstrate in Fig. 1 *C* that SRP receptor is also required. For this purpose, the 52-kD cytoplasmic domain of the SRP receptor α subunit (SRαf; Tajima et al., 1986) was removed by mild proteolysis of K-RM with trypsin yielding inactive K-RM (T-RM, Fig. 1 *C*, lane *4*). The activity of T-RM was recovered by reconstitution of SRP receptor accomplished by addition of the purified SRαf (Fig. 1 *C*, lane *4*). Thus, we can conclude that the translocation of full-length ppαF across mammalian RM requires both SRP and its receptor.

SRP is thought to bind to the signal peptide of a nascent protein after it has emerged from the ribosome (Walter et al., 1981). The SRP–ribosome–nascent chain complex is then targeted to the ER membrane by the specific interaction of SRP with SRP receptor (Walter and Blobel, 1981; Gilmore et al., 1982*a*, *b*; Meyer et al., 1982), and translocation is initiated. Signal recognition by SRP has been shown by direct cross-linking experiments but could only be demonstrated on nascent chains emerging from ribosomes and not after their release (Krieg et al., 1986; Kurzchalia et al., 1986). We were therefore interested in determining if the SRP and SRP receptor–dependent translocation of full-length ppαF also requires the functional involvement of the ribosome, even though elongation was no longer taking place.

We tested if the ribosome was required for translocation of full-length ppαF by three distinct criteria (Fig. 2). First, we found that preincubation of the translation extract containing ppαF with puromycin, an antibiotic that inhibits translation by releasing nascent chains from ribosomes,

abolishes translocation (Fig. 2 *A*, lane *3*). This suggested that the fraction of ppαF which could be translocated was ribosome associated as nonterminated peptidyl tRNA. We therefore asked if we could detect glycosylated pαF.3 that still retained the linkage to tRNA. For this purpose we precipitated the products after translocation with the cationic detergent CTABr, which at low pH will precipitate those polypeptides that are covalently attached to RNA (Hobden and Cundliffe, 1978). As shown in Fig. 2 *B*, ~50% of the pαF.3 was recovered in the pellet fraction (Fig. 2 *B*, compare lanes *2* [pellet] and *3* [supernatant] with the total reaction products in lane *1*). When the products were deacylated by treatment with base before CTABr precipitation, no pαF.3 was found in the pellet fraction (Fig. 2 *B*, compare lanes *4* and *5*), indicating that precipitation was indeed due to the presence of the covalently attached tRNA on these polypeptides. Thus a large fraction of the glycosylated pαF.3 remains attached to tRNA and presumably the ribosome. We envision these chains to be spanning the membrane such that the glycosylation sites are exposed on the lumenal side, yet the carboxy-terminal ends are still within (and protected from protease by) ribosomes on the cytoplasmic face of the membrane. We assume that the fraction of pαF.3 that was not CTABr precipitable has become deacylated during the incubation or subsequent manipulations.

A direct demonstration that all ppαF chains have to be ribosome associated to be translocation competent is shown in Fig. 2 *C*. We fractionated the translation reactions into a ribosomal pellet (Fig. 2 *C*, *RP*) and a postribosomal supernatant (Fig. 2 *C*, *PRS*) before the addition of K-RM and SRP. We observed translocation only when RM were incubated with the ribosome pellet fraction (Fig. 2 *C*, lanes *5* and *6*). No pαF.3 was observed when the postribosomal supernatant fraction was used, even at high SRP concentrations (Fig. 2 *C*, lanes *2* and *3*). Thus, the translocation of full-length ppαF across mammalian ER membranes must be dependent on the continued participation of the translation machinery. We can thus explain the apparent low efficiency of translocation under these conditions by the fact that the competent substrate (nonterminated ppαF) was present as a minor fraction of the translation products (~5% after 30 min of translation as determined by CTABr precipitation, data not shown). Given that 1–2% of the synthesized full-length ppαF was translocated, the reaction is in fact 20–40% efficient and thus comparable with other in vitro translocation systems. We found that prolonged incubation times will reduce the amount of nonterminated ppαF present in the translation extract. Thus, after a 1-h translation reduced or no translocation of full-length ppαF was observed (not shown). This may explain why Rothblatt and Meyer (1986) failed to detect translocation of full-length ppαF in their assays.

Lastly, we were interested in characterizing the energy requirements of the translocation reaction. After ppαF synthesis, translation was inhibited by cycloheximide as described above and small molecules (i.e., ATP, GTP, and creatine phosphate) were removed by gel filtration. No translocation of ppαF was observed upon addition of RM to the desalted fraction in the absence (not shown) or presence (Fig. 3, lane *5*) of an energy regenerating system. Translocation could be restored if ATP (1 mM, Fig. 3, lane *3*) was added back to the system. In addition, we found that the nonhydrolyzable ATP analogue, ATPγS, competed with ATP, causing half-

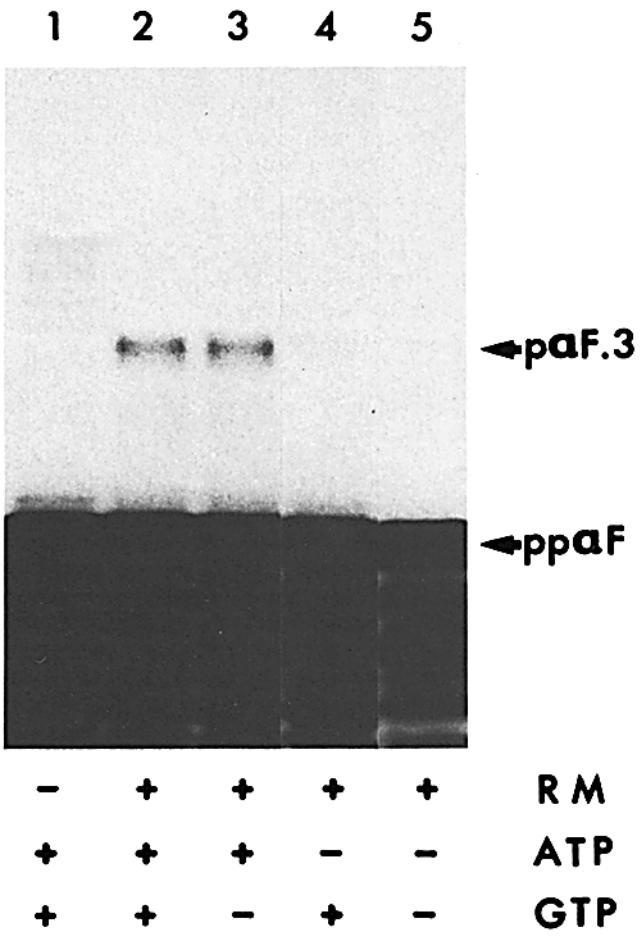

Figure 3. ATP hydrolysis is required for the ribosome-coupled translocation of full-length ppαF. Reactions were carried out as in Fig. 1 with the exception that small molecules were removed from the translation mixture by gel filtration. K-RM (5 eq/50 μl) and SRP (200 nM) were added to each reaction. A control reaction with no K-RM added is shown in lane *1*. ATP (1 mM, lanes *1–3*), GTP (100 μM, lanes *1*, *2*, and *4*), and creatine phosphate (8 mM to all reactions) were included as indicated. No translocation activity was observed when creatine phosphate was omitted. The microsomal vesicles were collected and analyzed as before. Competition experiments with nonhydrolyzable analogues of ATP (ATPγS) and GTP (GMPPNP) and with a GDP analogue (GDPβS) that cannot be kinased to the triphosphate were carried out under the same conditions (data not shown). All three analogues were purified by preparative TLC before use. In the presence of 1 mM ATP and 100 μM GTP, half-maximal inhibition of translocation was observed at 5 mM ATPγS, 500 μM GMPPNP, and 500 μM GDPβS (data not shown). (Since GMPPNP inhibits translocation we conclude that either an additional GTPase is required [which is not needed for short truncated products, Connolly and Gilmore, 1986] or that GMPPNP inhibits the ATPase described. The latter case is unlikely, since the corresponding adenine analogue, AMPPNP, showed only minor inhibition, even at 10 mM [not shown]. The effect of GDPβS could then be explained by either inhibition of this GTPase, or by inhibition at the stage of the GTP binding protein described [Connolly and Gilmore, 1986]. It is clear from these studies that the energy requirements are complex and that their complete understanding may have to await the biochemical description of the enzymes involved.)

maximal inhibition at 5 mM in the presence of 1 mM ATP (not shown). Thus translocation of full-length ppαF across mammalian RM requires ATP hydrolysis. In contrast, no translocation was observed if GTP (100 μM, Fig. 3, lane *4* or 1 mM, not shown) was added in the absence of ATP and no stimulation of translocation was observed if GTP was added in combination with ATP. While GTP by itself was not sufficient to promote translocation, it may still be required in addition to ATP, since a small amount of GTP could be present as a contaminant in the ATP solution or could be generated from residual GDP in the desalted extract. In fact, inhibitor studies with guanosine nucleotide analogues (not shown, but summarized in the legend to Fig. 3) hint at an additional requirement for GTP binding proteins.

Discussion

We have shown here that full-length ppαF can be efficiently translocated across mammalian RM membranes as long as the polypeptide chain is retained as peptidyl-tRNA on the ribosome. Thus, this reaction is distinct in its molecular requirements from the posttranslational translocation of ppαF across yeast RM from both the yeast translation system (Hansen et al., 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986) or the wheat germ translation system (Hansen et al., 1986), which can occur in the absence of SRP, SRP receptor, and ribosomes. Yeast RM appear to have a more flexible requirement for the form in which presecretory proteins are acceptable as translocation substrates. In molecular terms one can envision that the yeast analogue of the recently identified signal sequence receptor (Wiedmann et al., 1987) in the RM membrane can functionally engage with signal sequences on soluble preproteins, thereby bypassing a requirement for the ribosome, SRP, and SRP receptor (Walter, 1987). During translocation across mammalian RM, the signal sequence appears to be handed from SRP to the signal sequence receptor, once that the SRP–ribosome–nascent chain complex has been targeted via SRP receptor. Therefore, it appears that the mammalian signal sequence receptor is more stringent that its yeast counterpart, since it can functionally interact with signal peptides only when these have been properly "delivered" by the action of other components of the mammalian targeting machinery.

Our results indicate that signal recognition and targeting to the mammalian RM membrane by SRP occur only if the preprotein is seen in the context of the ribosome. Indeed, while SRP can be directly cross-linked to signal sequences that are part of the nascent polypeptide emerging from the ribosome (Kurzchalia et al., 1986; Krieg et al., 1986) no affinity of SRP for isolated signal peptides or preproteins released from the ribosome has yet been demonstrated. Since terminated ppαF is an efficient translocation substrate across yeast RM, our results rule out that the ribosome merely acts to hold the nascent polypeptide in a translocation competent state by sequestering the carboxy-terminal 40 amino acids within the ribosome and, thus, interfering with protein folding. Rather, the ribosome seems to be directly involved as a ligand required for signal recognition by SRP and possibly also required later for the formation of the ribosome–membrane junction.

It was previously suggested that the formation of a ribosome membrane junction requires GTP and involves a GTP binding protein, but that no additional energy input is required to translocate small (86 amino acids) nascent preprolactin polypeptide chains (Connolly and Gilmore, 1986). Although we have not been able to demonstrate unambiguously a GTP requirement for the translocation of full-length ppαF, our data are not in disagreement. However, we clearly demonstrated that ATP hydrolysis is required, as was previously found for posttranslational translocation of soluble ppαF across yeast RM (Hansen et al., 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986) and suggested for the insertion of a fragment of the glucose transporter protein into mammalian RM (Mueckler and Lodish, 1986*b*). We speculate that longer polypeptide chains have a tendency to fold, thereby making the signal peptide inaccessible. Consequently, additional energy may be necessary to unfold the substrate before translocation. This notion is further supported by the finding that optimal translocation occurs at high SRP concentrations, which is consistent with the idea that the nascent chain is in equilibrium between having a buried or an exposed signal peptide, and that high SRP concentration drives this equilibrium to the exposed state. A correlation between nascent chain length and the concentration of SRP required for efficient targeting has also been directly demonstrated for bovine preprolactin (Siegel, V., and P. Walter, manuscript submitted for publication).

Finally we wish to comment on the nomenclature currently used. We have demonstrated here that the mammalian translocation machinery requires that nascent secretory proteins be attached to the ribosome as peptidyl-tRNA. Previously, elongation independent processes have been collectively referred to as "posttranslational". Thus, while targeting and translocation of full-length preproteins across mammalian RM are independent of ongoing elongation, they are not posttranslational events. No translocation of these chains would occur if the final step in translation, termination, had already taken place. This is in contrast to the translocation of ppαF and other yeast secretory proteins (Hansen et al., 1986; Hansen, W., and P. Walter, manuscript submitted for publication) which can be translocated across yeast RM from a soluble pool in a truly posttranslational mode. We therefore wish to distinguish between these two processes, fundamentally different in their molecular requirements, and propose the term "ribosome-coupled translocation" for the events described here for mammalian RM. We suggest that SRP, which is required in this reaction, has evolved primarily as an adapter between the ribosome and the membrane. Most proteins that have been described to be translocated across mammalian RM in the absence of protein synthesis fall into this category. In all cases a ribosome-dependence has been noted and their translocation has been improperly referred to as posttranslational (Caulfield et al., 1986; Mueckler and Lodish, 1986*a*, *b*; Perara et al., 1986; Chao et al., 1987; see also Fig. 7 in Hansen et al., 1986). The only known exceptions are a few small peptides, prepromelittin (Zimmermann and Mollay, 1986), m13 precoat protein (Watts et al., 1983), and GLa peptide (Schenstedt and Zimmermann, 1987). These peptides appear to be substrates for posttranslational translocation across mammalian RM with no ribosome, SRP, and SRP receptor requirement. Due to their small size and/or particular structure, it is possible that they use a different translocation mechanism with different molecular requirements.

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